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Autophagy Negatively Regulates Keratinocyte Inflammatory Responses via Scaffolding Protein p62/SQSTM1

Hye-Mi Lee,*† Dong-Min Shin,*‡ Jae-Min Yuk,*† Ge Shi,‡ Dae-Kyoung Choi,‡ Sang-Hee Lee,†‡ Song Mei Huang,‡† Jin-Man Kim,†‡ Chang Deok Kim,†‡‡ Jeung-Hoon Lee,‡*,† and Eun-Kyeong Jo,∗*,†*


As the primary component of the epidermal barrier, keratinocytes play an essential role in innate immune responses to exogenous pathogens. TLRs are key pattern recognition molecules in innate immunity that activate the NF-κB pathway, resulting in the production of antimicrobial immune and proinflammatory mediators (1). Whereas several TLRs, including TLR2, 3, and 5, are constitutively expressed, others are inducible in human keratinocytes (2). Excessive activation of TLRs in keratinocytes has been reported in chronic inflammatory conditions, including psoriasis (3). Notably, TLR2 has been reported to be upregulated in psoriatic skin and cells from patients with psoriatic arthritis (4, 5), suggesting that it may play an important role in chronic cutaneous inflammation. Recent studies have highlighted the potential of TLR2-targeting therapies to blunt inflammatory responses without interfering with microbial recognition (6).

Autophagy is a major intracellular process in which lysosomes degrade protein aggregates, cytoplasmic organelles, and aged proteins (7, 8). Several TLR ligands, including a subset of TLR7 ligands and the TLR4 ligand LPS, are potent inducers of autophagy in macrophages (7, 9, 10). Recent studies have implicated autophagy in the regulation of inflammatory immune responses (11). The protein p62 (also known as SQSTM1) has been reported to link the recognition of polyubiquitinated protein aggregates to the autophagy machinery (12). As a signaling adaptor, p62 has been implicated in the activation of the transcription factor NF-κB through interaction with TRAF-associated factor (TRAF) 6 (13). Additionally, p62 interacts with the autophagic effector protein LC3 and is degraded through an autophagy–lysosome pathway (14, 15). A recent study showed that p62 protein accumulates in autophagy-defective tumor cells, and that the modulation of p62 expression by autophagy is a key factor in oncogenesis (16). These data suggest that p62 accumulation associated with the dysregulation of autophagy is involved in increased inflammation and tumorigenesis. However, the role and regulation of p62 in human keratinocytes have not been characterized.

In this study, we demonstrate that stimulation of human keratinocytes with TLR2/6 ligands strongly induces p62 expression and activates autophagy through NADPH oxidase (NOX)-dependent generation of reactive oxygen species (ROS). MyD88 and TRAF6, signaling molecules that participate in TLR-dependent activation of NF-κB signaling, were necessary for the induction of p62 expression and autophagy. Moreover, inhibition of autophagy significantly increased the accumulation of p62 (which is responsible for the activation of NF-κB signaling), the expression of cathelicidin/LL-37, and the production of inflammatory cytokines. Notably, skin from patients suffering from psoriasis displayed increased p62 and cathelicidin expression, indicating that the potential of TLR2-targeting therapies to blunt inflammatory responses without interfering with microbial recognition (6).

Abbreviations used in this article: AD, atopic dermatitis; Baf-A1, bafilomycin A1; Cdk, cyclin-dependent kinase; 3-MA, 3-methyladenine; NAC, N-acetylcysteine; NOX, NADPH oxidase; ROS, reactive oxygen species; shAtg5, short hairpin RNA specific to hAtg5; shBecn1-1, short hairpin RNA specific to hBecn1-1; shNS, nonspecific control short hairpin RNA; siRNA, small interfering RNA; TRAF, TNFR-associated factor.

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p62 accumulation may contribute to the pathogenesis of this chronic inflammatory skin disorder.

Materials and Methods

Cell cultures

Human primary keratinocytes and HaCaT cells were cultured and maintained, as described previously (17). Briefly, foreskin epidermal keratinocytes were cultured as keratinocyte serum-free medium supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Life Technologies/Life Technologies, Eggenstein, Germany). HaCaT cells were cultured in DMEM (Life Technologies-BRL, Gaithersburg, MD), supplemented with 10% FCS (Invitrogen, San Diego, CA), 1% L-glutamine, penicillin (50 U/L; Life Technologies-BRL), and streptomycin (50 mg/mL; Life Technologies-BRL). The study protocol was approved by the Institutional Research Board of Chungnam National University College of Medicine. Written informed consent was obtained from each participant.

Reagents, DNA, and Abs

MALP-2, a diacylated lipopeptide, was purchased from Invitrogen (San Diego, CA). N-acetylcyesteine (NAC), diphenylene iodonium (DPI), allopurinol, and wortmannin were obtained from Calbiochem (San Diego, CA), and 3-methyldiazole (3-MA), baflomycin A1 (Baf-A1), rapamycin, LPS (Escherichia coli 0111:B4), and DMSO were from Sigma-Aldrich (St. Louis, MO). psiRNA-hLSKGFp2eo plasmids specific for HTLR2 and hTLR4 were purchased from InvivoGen. Cells were transfected using Lipofectamine 2000, as suggested by the manufacturer (Invitrogen, Carlsbad, CA). Anti–phospho-Akt (Ser17/Thr22), anti–phospho-ERK1/2 (Thr202/Tyr204), Abs were obtained from Cell Signaling Technology, an anti-LC3 Ab from Novus Biologicals (Littleton, CO), and Abs specific for p62/hp62/SQSTM1 (H-290), cyclin D1 (H-295), cyclin-dependent kinase (Cdk) 4 (H-22), Beclin-1 (B-290), hbeclin-1 (NM_001142298), were supplied by Sigma-Aldrich as glycerol stocks. Reagents, DNA, and Abs

Transmission electron microscopy

Transmission electron microscopy was performed, as described previously (18). HaCaT cells were fixed in 4% paraformaldehyde at 4˚C for 10 min. They were then permeabilized with 0.01% Triton X-100, before being blocked with 10% BSA for 1 h. Cells were stained with primary Ab (rabbit anti-human LC3, diluted 1:400; Santa Cruz Biotechnology) and secondary Ab (anti-rabbit IgG-Alexa488) for 1 h. Unbound Ab was removed with PBS, and cells were imaged using a Zeiss LMS510 META confocal microscope (Zeiss).

Transmission electron microscopy

Transmission electron microscopy was performed, as described previously (18). Briefly, MALP-2–stimulated human primary keratinocytes were fixed with 3% formaldehyde and 2% glutaraldehyde (Electron Microscopy Sciences, Road Hatfield, PA) in 0.1 M sodium cacodylate buffer (pH 7.4; Ted Pella, Redding, CA) for 1 h, then washed with 0.1% cacodylate buffer and postfixed with 1% osmium tetroxide (Electron Microscopy Sciences) and 0.5% potassium ferricyanide in cacodylate buffer for 1 h. Cells were then dehydrated with increasing concentrations of ethanol, and infiltrated with Epon-Araldite resin at 80˚C for 24 h. Ultrathin sections (70–80 nm) were obtained using an ultramicrotome (RMC MT6000-XL). Sections were contrasted with uranyl acetate and lead citrate and viewed using a Zeiss EM 900 transmission electron microscope (Zeiss).

Immunohistochemistry

The subcellular localization of human p62 in tissue sections was determined through immunohistochemical staining, performed as described previously (17). Biopsies tissue obtained from patients with chronic atopic dermatitis (AD), as well as five healthy controls, was fixed in formalin, embedded in paraffin, and sectioned to a thickness of 3 μm. After overnight incubation with the primary Ab (rabbit polyclonal p62 Ab, diluted 1:400; Abgent) at 4˚C, tissue sections were incubated with the substrate 3,3′-diaminobenzidine (Dako) to generate a colored signal. They were lightly counterstained with Mayer’s hematoxylin and mounted using ImmunoMount (Thermo Shandon, Pittsburgh, PA). Negative control sections were treated with serum from nonimmunized rabbits (Dako) in place of primary Ab. Cells displaying cytoplasmic staining were considered to be positive. A single pathologist examined all the immunostained tissue sections.
Statistical analysis

All data are presented as the mean ± SD of independent experiments. Statistical analysis was performed using a paired Student t test with Bonferroni correction or, for multiple comparisons, ANOVA. The p values <0.05 were deemed to be statistically significant.

Results

TLR2/6 stimulation activates autophagy in primary human keratinocytes

It has previously been shown that TLR ligands stimulate the formation of autophagosomes and autophagolysosomes (7, 9, 10). However, it is not known whether TLR agonists activate autophagy in primary human keratinocytes. To determine whether TLR2/6 stimulation activates autophagy in human keratinocytes, we stimulated primary human keratinocytes with MALP-2 (100 ng/ml) and measured the formation of organelles displaying punctate LC3 staining (autophagosomes) using endogenous LC3 as an autophagic marker. As shown in Fig. 1A, MALP-2 induced autophagy in primary human keratinocytes, with peak activation occurring at 36 h. In addition, posttranslational modification of LC3-I, yielding LC3-II (19), was assessed in MALP-2–stimulated human keratinocytes. Significant increases in the LC3-II:LC3-I ratio were observed in primary human keratinocytes (Fig. 1B) and HaCaT cells (Supplemental Fig. 1A) after MALP-2 stimulation. LC3 autophagosome formation was significantly inhibited in cells pretreated with 3-MA or wortmannin, specific inhibitors of autophagy (20, 21) (Fig. 1C). Additionally, MALP-2–induced punctate LC3 staining was significantly reduced in cells in which Beclin-1 or Atg5 was silenced through transfection with a specific lentiviral shRNA (Fig. 1D).

Colocalization of LC3 and lysosomes (the latter stained with LysoTracker) was significantly increased in primary human keratinocytes following MALP-2 stimulation (Fig. 1E). In primary keratinocytes, the ability of MALP-2 to increase the LC3-II:LC3-I ratio (measured by Western blotting) was attenuated by pretreatment with 3-MA (Fig. 1F). Furthermore, pretreatment with the vacuolar H+-ATPase inhibitor Baf–A1, which prevents lysosomal...
duced p62 mRNA expression. Transfection of HaCaT cells with a siRNA specific for hTLR2, but not one targeted to hTLR4, significantly inhibited MALP-2–induced p62 expression (Fig. 2C). Thus, MALP-2 actively induces p62 expression in primary human keratinocytes via TLR2.

**NOX-dependent generation of ROS is required for the induction of p62 expression and activation of autophagy in TLR2/6-stimulated primary human keratinocytes**

ROS derived from superoxide play multiple roles in innate immune responses, including in microbial killing, the regulation of apoptosis, cytokine production, and gene expression, and the activation of antibacterial autophagy (24, 25). We first examined whether MALP-2 increased the intracellular generation of superoxide in human keratinocytes using the oxidative fluorescent dye dihydroethidium (DHE). As shown in Fig. 3A, MALP-2–induced superoxide production was detected within 30 min, peaking 1 h after stimulation. We next examined whether MALP-2–dependent ROS generation was required for p62 expression and the activation of autophagy. Treatment of keratinocytes with either the general ROS scavenger NAC or the NOX inhibitor DPI, but not the xanthine oxidase inhibitor allopurinol, significantly and dose dependently attenuated MALP-2–induced p62 expression (Fig. 3B). Previously, it has been shown that HaCaT cells express significant amounts of the gp91phox homologs NOX2 and NOX4, but only very low levels of NOX1 mRNA (26). Thus, we further investigated the roles of NOX2 and NOX4 in the MALP-2–mediated induction of p62. Silencing NOX2 or NOX4 using specific siRNAs significantly attenuated MALP-2–induced p62 expression in HaCaT cells (Fig. 3C).

The MALP-2–induced formation of autophagosomes displaying punctate LC3 expression (Fig. 3D) and increase in the LC3-II:LC3-I ratio (Fig. 3E) was dose dependently abrogated by pretreatment with NAC or DPI, but not allopurinol. These data suggest that TLR2/6-dependent ROS generation also plays a role in the activation of autophagy in keratinocytes. Transfection of HaCaT cells with siRNAs specific to hNOX2 and hNOX4 significantly inhibited autophagosome formation in response to MALP-2 (Fig. 3F). These data suggest that the NOX-dependent generation of ROS is critically involved in MALP-2–mediated p62 expression and autophagy in keratinocytes.

**TLR4 stimulation also leads to ROS-dependent activation of autophagy and p62 expression in keratinocytes**

Having established the role of TLR2/6 in the induction of autophagy, p62 expression, and ROS generation in human keratinocytes, we examined whether other TLR ligands function similarly in keratinocytes. Although previous studies showed that the TLR4 agonist LPS/endotoxin induced autophagy in macrophages (7, 9), a similar response has not been demonstrated in keratinocytes. As TLR4 was shown to be constitutively and inducibly expressed in HaCaT cells (2), we first examined punctate LC3 expression and LC3-II/LC3-I levels in HaCaT cells after LPS stimulation. Treatment of HaCaT cells with LPS robustly increased punctate LC3 expression and LC3-II band intensities at 18 h, indicating activation of autophagy (Fig. 4A, 4B). The marked increase in punctate LC3 staining was largely abrogated by pretreatment with 3-MA (Fig. 4A), a novel inhibitor of autophagy activation (20, 21). TLR4/ LPS stimulation significantly increased the endogenous expression of p62 and LL-37 in HaCaT cells (Fig. 4C, p62 and LL-37 mRNA; Fig. 4D, p62 protein). We further showed that LPS stimulation robustly activated ROS generation in HaCaT cells in a time-dependent manner, and that the peak was observed within 30 min of stimulation (Fig. 4E). Blocking ROS production using the antioxidants NAC and DPI dose-dependently inhibited LC3-II expression (Fig. 4F). Additionally, transfection of HaCaT cells

**FIGURE 2.** TLR2/6 stimulation induces the expression of p62/ SQSTM1. A and B, Primary human keratinocytes were stimulated with MALP-2 (2) and measured p62 mRNA and protein expression by RT-PCR and Western blotting, respectively. Endogenous p62 mRNA and protein expression was significantly increased by 6 h. Images are representative of three independent experiments, all yielding similar results (top). Data represent the mean ± SD of three independent experiments (bottom). ***p < 0.001 (versus control).
with siRNAs specific for hNOX2 and hNOX4 significantly inhibited LPS-induced p62 expression (Fig. 4G). These data collectively show that the activation of TLR4, as well as TLR2/6, significantly upregulated NOX-dependent ROS generation, which contributes to autophagy activation and the expression of p62 and LL-37 in human keratinocytes.

**MylD88 and TRAF6 are required for p62 expression and the activation of autophagy in keratinocytes**

It was previously shown that the adaptor protein p62 selectively interacts with the TRAF domain of TRAF6 and plays an important intermediary role in the activation of NF-κB by TNF-α or IL-1 (27). Additionally, MylD88 plays a role in TLR4- or TLR7-induced activation of autophagy (7, 10). We therefore examined whether MALP-2–dependent activation of autophagy was mediated by MylD88 and/or TRAF6, both of which contribute to TLR signaling. When HaCaT cells were transfected with siRNAs specific for hMylD88 and hTRAF6, MALP2/6-dependent p62 protein (Fig. 5A) and mRNA expression (Fig. 5B) were significantly reduced. Furthermore, the MALP-2–induced increases in LC3-positive autophagosome formation (Fig. 5C) and the LC3-II:LC3-I ratio (Fig. 5D) were significantly reduced in HaCaT cells transfected with siRNAs specific for hMylD88 or hTRAF6. These data suggest that both MylD88 and TRAF6, key signaling intermediates in TLR-dependent signaling, are involved in the activation of autophagy in human keratinocytes.

**The autophagy pathway negatively regulates TLR2/6- and/or TLR4-induced inflammatory responses and p62 expression in keratinocytes**

Accumulating evidence suggests that autophagy contributes to the regulation of cellular inflammation (28). Recently, it has been reported that autophagy-linked genes are involved in susceptibility to Crohn’s disease (29), and that autophagy defects exacerbate endotoxin-induced activation of the inflammasome (30). We next examined whether the activation of autophagy plays a role in the regulation of inflammatory responses and NF-κB activation in primary keratinocytes. To test this, primary human keratinocytes were pretreated with 3-MA or wortmannin, two inhibitors of the kinase PI3K that is required for the activation of autophagy (31), or transfected with shBeclin-1 or shAtg5 prior to MALP-2 stimulation. Notably, the secretion of TNF-α and IL-6 was dose dependently increased in primary human keratinocytes pretreated with 3-MA or wortmannin (Fig. 6A). Primary human keratinocytes transfected with shBeclin-1 or shAtg5 displayed significant increases in MALP-2–induced production of the proinflammatory cytokines TNF-α and IL-6 (Fig. 6B). Additionally, MALP-2–dependent NF-κB reporter gene activity was significantly increased in HaCaT cells pretreated with 3-MA or wortmannin (Supplemental Fig. 2A) and primary human keratinocytes transfected with shBeclin-1 or shAtg5 (Fig. 6C).

Next, the role of autophagy in regulating p62 expression was determined in human keratinocytes. Blockade of the autophagy pathway negatively regulates TLR2/6- and/or TLR4-induced inflammatory responses and p62 expression in keratinocytes. Blockade of the autophagy pathway negatively regulates TLR2/6- and/or TLR4-induced inflammatory responses and p62 expression in keratinocytes.
pathway using 3-MA or wortmannin significantly increased MALP-2–induced p62 protein expression in primary human keratinocytes (Fig. 6D). Transfection of primary human keratinocytes with shBeclin-1 or shAtg5 significantly increased the induction of p62 protein expression following MALP-2 stimulation (Fig. 6E). We further characterized the role of autophagy in TLR4-stimulated keratinocytes. Consistent with the results of TLR2/6 stimulation, pretreatment of HaCaT cells with 3-MA or wortmannin (30 or 50 μM), DPI (20 or 50 μM), or allopurinol (Allo; 0.1 or 1 mM), HaCaT cells were treated with LPS for 18 h and analyzed for LC3 protein expression. In Western blot analysis of p62 levels in HaCaT cells transfected with siRNAs specific for hnox2 (siNOX2) and hnox4 (siNOX4), or siNS, and then treated with LPS (100 ng/ml). Images are representative of three independent experiments, all yielding similar results (B–D, F, G). L, LPS; U, untreated.

**FIGURE 4.** TLR4-induced expression of p62 and activation of autophagy in human keratinocytes are dependent on ROS. A, Immunofluorescence analysis of LC3 punctate expression. HaCaT cells were stimulated with LPS (100 ng/ml) in the absence or presence of 3-MA (2 h, 10 μM) for 18 h. Cells were stained with DAPI and anti-LC3–Alexa 488 Ab (experimental conditions as described in Fig. 1C). Data represent the mean ± SD of three independent replicates, each comprising at least 250 cells scored in five random fields. B–D, HaCaT cells were stimulated with LPS (100 ng/ml) for the indicated periods of time and analyzed for LC3 and p62 protein expression (B and D, Western blot analysis) and p62 and LL-37 mRNA expression (C, semiquantitative RT-PCR). E, Measurement of intracellular superoxide generation through fluorescence analysis. HaCaT cells were stimulated with LPS for the indicated periods of time. Cells were then incubated with DHE (10 μM) for 15 min and analyzed for superoxide production by confocal microscopy. Data represent the mean ± SD of three independent experiments. F, Western blot analysis of LC3 expression. Following preincubation for 45 min with medium containing NAC (30 or 50 mM), DPI (20 or 50 μM), or allopurinol (Allo; 0.1 or 1 mM), HaCaT cells were treated with LPS for 18 h and analyzed for LC3 protein expression. G, Western blot analysis of p62 levels in HaCaT cells transfected with siRNAs specific for hnox2 (siNOX2) and hnox4 (siNOX4), or siNS, and then treated with LPS (100 ng/ml). Images are representative of three independent experiments, all yielding similar results (B–D, F, G). L, LPS; U, untreated.

Recent studies have defined the roles of p62 in NF-κB activation and oncogenesis in autophagy-defective tumor cells (16), suggesting its involvement in inflammatory responses and tumorigenesis. To further explore the role of p62 in inflammatory responses in human keratinocytes, p62 was knocked down in HaCaT keratinocytes through transfection with a specific siRNA. Silencing p62 in this way led to significant decreases in the expression of TNF-α and IL-6 mRNA and protein in response to MALP-2 stimulation (Fig. 7A, 7B). The induction of LL-37 mRNA expression in HaCaT cells by TLR2/6 was suppressed by transfection with siRNA specific to hp62 (si-p62; Fig. 7A).

Following TLR2/6 stimulation, the activation of MyD88-dependent TRAF6 signaling leads to the activation of TGF-activator kinase 1, which phosphorylates the IKK complex (32). The IKK complex, in turn, phosphorylates IκB proteins, resulting in their ubiquitination and degradation (32). This key event triggers the translocation of NF-κB to the nucleus, where it mediates the transcription of target genes (32). To further investigate the underlying mechanisms by which p62 modulates TLR2/6–dependent inflammatory responses, we examined whether p62 played a role in IκKα/β phosphorylation and IκBα degradation in response to MALP-2. As shown in Fig. 7C, MALP-2 stimulation led to the time-dependent phosphorylation of IκKα/β and concomitant degradation of IκBα in human keratinocytes transfected with shNS. The transduction of primary keratinocytes with shRNA specific for hp62/SQSTM1 prior to MALP-2 stimulation markedly inhibited the phosphorylation of IκKα/β and degradation of IκBα in human keratinocytes (Fig. 7C). Moreover, the silencing of p62 in HaCaT cells by p62 siRNA significantly inhibited NF-κB–luciferase reporter gene activity in response to MALP-2 treatment (Fig. 7D).

We next examined whether the role of p62 in mediating inflammatory responses was confined to TLR2/6 responses. Silencing of p62 led to the significant inhibition of LPS-induced expression of TNF-α, IL-6, and LL-37, and NF-κB reporter gene activity, in HaCaT cells (Supplemental Fig. 3A–C). Thus, p62 appears to play
critical roles in TLR2/6- and/or TLR4-induced inflammatory responses through activation of NF-κB signaling.

We further investigated whether p62 plays roles in MALP-2–dependent cell proliferation, cyclin D1/Cdk4 expression, and the activation of Akt and ERK1/2 in human keratinocytes. As shown in Fig. 7E, MALP-2 stimulation resulted in a substantial increase in keratinocyte proliferation, as measured in a Cell Counting Kit-8 assay. Silencing p62 expression led to the time-dependent inhibition of keratinocyte proliferation (Fig. 7E). Additionally, transduction of human primary keratinocytes with shRNA specific for p62 efficiently reduced the expression of cyclin D1/Cdk4 and phosphorylation of Akt (Fig. 7F), suggesting that p62 plays key roles in keratinocyte proliferation and cell cycle progression. When primary keratinocytes were transduced with shRNA specific for p62, but not shNS, the phosphorylation of ERK1/2 was significantly reduced (Fig. 7G). These data collectively show that p62 plays important regulatory roles in keratinocyte inflammatory responses through the activation of IKK complex–NF-κB signaling, and in keratinocyte proliferation via cyclin D1-/Cdk4-, Akt-, and ERK1/2-dependent signaling pathways.

Epidermal expression of p62 and LL-37 is up-regulated in patients with psoriasis, but not AD

Based on the roles of p62 in the regulation of inflammatory responses and proliferation, we next extended our analyses to determine whether p62 levels are elevated in inflammatory skin diseases.
orders. To test this hypothesis, we performed comparative analyses of epidermal p62 expression in plaque psoriasis and eczema. Psoriasis is characterized by increased inflammation and the release of antimicrobial peptides, as well as hyperproliferation caused by disturbed differentiation of keratinocytes (3, 33). Eczemas represent a group of etiological and regional inflammatory skin disease variants characterized by pruritic, papulovesicular, and weeping dermatitis (34). As eczemas are a diverse group of disorders, we focused on the p62 expression profile in AD, an inflammatory skin disease that is typically familial, and one that is associated with IgE reactivity, Th2 predominance, and mast cell hyperactivity (35, 36).

To examine the role of p62 and cathelicidin LL-37 in inflammatory skin conditions, we measured the expression of p62 and LL-37 in the epidermis of biopsy samples obtained from psoriasis and AD patients and healthy subjects. Immunohistochemical staining revealed high numbers of p62-expressing keratinocytes in the skin of all psoriatic patients studied (35 of 35), but in no AD patients (0 of 22) and only a minority of healthy controls (5 of 16). The p62 expression was primarily detected in the cytoplasm in samples of psoriatic skin (Fig. 8A). As shown in Fig. 8B, RT-PCR analysis of mRNA extracted from patient skin samples revealed significantly higher expression of p62 and LL-37 in psoriasis patients than in AD patients or healthy controls. These results indicate that lesional psoriatic skin displays marked upregulation of p62 and cathelicidin in the pathogenesis of psoriasis in humans.

Discussion

In human keratinocytes, TLR signaling robustly activates autophagy and p62 expression. In cells in which autophagic function was lost, inflammatory responses were profoundly exacerbated. Expression of p62 increased in parallel with these changes. In MALP-2–treated cells, knockdown of p62 not only reduced proinflammatory cytokine production, but also inhibited proliferation. In psoriatic skin, but not in skin from AD patients, p62 expression was significantly increased. These findings suggest the involvement of autophagy in the regulation of excessive cutaneous inflammation and implicate the upregulation of p62 in the pathogenesis of psoriasis.

Accumulating evidence suggests that the recognition of multiple pathogen-derived molecules can activate autophagy, principally in mononuclear phagocytes (7, 9, 10). TLR7 and TLR4 ligands have been reported to be potent inducers of autophagy in macrophages (7, 9). Additionally, stimulation of TLR2 through zymosan treatment can induce autophagy, specifically the formation of phagosomes and their fusion with lysosomes (37). However, it has not been detailed previously whether TLRs activate autophagy in human keratinocytes. Our data provide evidence that TLR signaling robustly activates autophagy in human keratinocytes. Electron microscopic imaging showed the rapid formation of autophagosomes and autolysosomes in human keratinocytes following TLR2/6 stimulation. Moreover, cells stimulated with MALP-2 displayed clear autophagic features, including punctate LC3 staining and colocalization of LC3 and lysosome markers. In addition,

FIGURE 7. The stimulation of inflammatory responses and cell proliferation in human keratinocytes by MALP-2 is dependent on p62/SQSTM1. A, B, and D, HaCaT cells were transfected with siNS or a siRNA specific for hp62 (sip62). Following transfection, cells were stimulated with MALP-2 (100 ng/ml) for 6 h (A, D, RT-PCR analysis and luciferase assay, respectively) or 18 h (B, ELISA analysis). A, Total RNA was isolated from cells and subjected to semi-quantitative RT-PCR analysis to measure TNF-α, IL-6, LL-37, and p62/SQSTM1 mRNA levels. B, Supernatants were harvested, and the levels of TNF-α and IL-6 were assessed by ELISA. D, NF-κB-luc reporter gene analysis. HaCaT cells were transiently transfected with a NF-κB reporter construct (experimental conditions as described in Fig. 6C) prior to stimulation with MALP-2 for 6 h, and then assayed for luciferase activity. C, F, and G, Primary human keratinocytes transduced with shNS or a shRNA specific for p62/SQSTM1 (shp62) were stimulated with MALP-2 for the indicated periods of time (C, F, G). They were then analyzed, by Western blotting, for levels of phospho-IKKα/β, IkB-α (C), phospho-Akt, cyclin D1, Cdk4 (F), and phospho-ERK1/2 (G). HaCaT cells were transiently transfected with siNS or sip62, stimulated with MALP-2, and subjected to a Cell Counting Kit-8 assay (F). Data are representative of three independent experiments, all yielding similar results (A, C, F, G). Data represent the mean ± SD of five independent experiments (B, D, E). Transfection efficiency was assessed by RT-PCR (D, inset). ***p < 0.001 (versus control). M, MALP-2; U, untreated.
MALP-2 stimulation significantly increased levels of lipid-bound LC3-II. During autophagy, the autophagic marker LC3 is cleaved and covalently conjugated to phosphatidyl-ethanolamine, yielding LC3-II, which is specifically targeted to elongated, Atg12-/Atg5-associated autophagosome precursors, thereafter remaining associated with autophagosomes (38, 39). Moreover, our data show that TLR4/LPS stimulation further induces autophagy in keratinocytes (see Fig. 4). Thus, our data suggest that the stimulation of innate immunity-associated TLRs in primary human keratinocytes actively induces autophagosome formation and regulates the maturation of autophagolysosomes.

We found p62 expression and the activation of autophagy to be dependent on NOX-mediated ROS generation and MyD88- and TRAF6-mediated signaling. Indeed, p62 mediates the TNF-α- and IL-1-dependent activation of NF-κB by interacting with the specific adapters receptor-interacting protein and TRAF6 (27, 40). Recent studies demonstrated that ROS generation contributes to the activation of autophagy, TLR activation, and phagocytosis in PMA-stimulated neutrophils (41). Similar to a previous study (25), we found that NOX activity and ROS generation played important roles in the activation of autophagy in primary human keratinocytes. Moreover, MyD88 has been previously implicated in TLR4- or TLR7-induced autophagy (7, 9, 10). Both key adaptor molecules in TLR signaling, MyD88 and TIR-domain–containing adapter-inducing IFN-β, modulate the interaction between Bcl-2 and Beclin-1, thereby actively triggering and regulating autophagy (10). Together, these data implicate innate immune signaling and key adaptor molecules in the activation of autophagy pathways, not only in leukocytes, but also in nonleukocytic cells.

In this study, we have demonstrated that the inhibition of autophagy results in significantly increased inflammatory cytokine production and p62 expression in primary human keratinocytes. Enhanced inflammatory responses and increased cell proliferation were observed in keratinocytes treated with compounds that interfere with early (3-MA) and late (Baf-A1) autophagic processes or with siRNA targeted to genes essential for autophagy (hBeclin-1, hAtg5). Recently, it was shown that a defect in autophagy potentiated RIG-like receptor signaling and increased IFN secretion caused by ROS generated by dysfunctional mitochondria (42). Evidence suggests that autophagy acts to regulate cellular homeostasis and signaling by altering the availability of key adaptor proteins, such as p62 (43) and Nbr1 (12). Notably, p62 is itself an important intracellular target for autophagy: its cytosolic accumulation represses inclusion body formation (43). Previous studies showed that the inhibition of autophagy leads to the accumulation of p62 in transformed cells, thus compromising the clearance of ubiquitinated proteasomal substrates (44). Together, these data suggest that the pathways controlling the activation of autophagy tightly control p62 levels to prevent homeostasis from being disrupted during inflammatory responses.

Our data further show that knockdown of p62 using a specific siRNA reduced the expression of inflammatory cytokine genes and cathelicidin, cell proliferation (as assessed by measuring cyclin D expression), and NF-κB promoter activity in keratinocytes. Earlier studies showed that dominant-negative mutants and the downregulation of p62 using an antisense plasmid markedly inhibit the activation of NF-κB by TNF-α, suggesting a critical role for p62 (40). It has been reported that cytosolic p62/SQSTM1 expression is induced by Ras, is required for IκB kinase activation through polyubiquitination of TRAF6, and contributes to the development of Ras-induced lung adenocarcinomas in murine models (45). Additionally, sustained p62 expression accompanying defective...
autophagy results in altered NF-κB regulation and gene expression, thus favoring tumorogenesis (16). As an atypical protein kinase C scaffold protein, p62 plays an important role in the activation of NF-κB in response to IL-1, TNF-α, and receptor activator for NF-κB ligand through the assembly of an aPKC/p62/TRAf6 multi-protein complex (46). Notably, our data demonstrate that p62 expression is crucial for the TLR-dependent activation of IKKα/β and degradation of IκBα in human keratinocytes (see Fig. 7). Thus, the cytosolic accumulation of p62/SQSTM1 may trigger TLR-induced inflammatory responses in keratinocytes through modulation of IKK–NF-κB signaling.

Both psoriasis and AD are common chronic and relapsing inflammatory skin diseases (33–35). Psoriasis is an inflammatory skin disease characterized by increased levels of TLRs 1, 2, 4, 5, and 9 (reviewed in Ref. 3). It has been suggested that upregulated TLR expression in psoriatic skin contributes to disease pathophysiology by increasing the local production of antimicrobial peptides, and stimulating cutaneous immune responses and epidermal hyperproliferation (3, 33). In contrast, monocytes from AD patients displayed significantly impaired TLR2-mediated production of the proinflammatory cytokines IL-1β and TNF-α (47). Additionally, the immunologic factors involved in the pathogenesis of AD are associated with upregulated IgE reactivity, and increased numbers of mast cells, eosinophils, and CD4-activated Th2 cells (34–36). Importantly, we found that the expression of p62 and cathelicidin was higher in psoriatic skin lesions than in lesions from AD patients or skin from healthy controls (see Fig. 8). The current data partly corroborate the findings of previous studies, which showed that psoriatic skin contained high amounts of the antimicrobial peptide cathelicidin/LL-37 (48). LL-37 potently activates plasmacytoid dendritic cells to produce type 1 IFNs by binding nonstimulatory self-DNA and causing it to adopt an aggregated and condensed structure (49). Several previous studies further showed that levels of various antimicrobial peptides, including LL-37 and human β-defensin 2, are significantly decreased in acute and chronic lesions from AD patients (50). The depressed expression of innate immune effectors and antimicrobial peptides may contribute to the increased susceptibility of AD patients to bacterial and viral infections (3, 51, 52). Together, p62 and cathelicidin may play important roles in the homeostatic regulation of cutaneous inflammatory responses, especially in psoriasis. Future studies should clarify the detailed molecular mechanism(s) by which enhanced p62 expression exacerbates psoriatic inflammatory responses by breaking innate tolerance and driving autoimmunity.

The current findings demonstrate a major role for autophagy in the regulation of keratinocyte inflammation and provide new insights into the mechanisms by which TLR signaling activates autophagy in human keratinocytes. TLR innate signaling activation in keratinocytes triggers a simultaneous induction of innate inflammatory responses and autophagy pathways. Importantly, the autophagy pathways were found to play a key role in fine tuning of inflammatory responses in keratinocytes. The ability of the inhibition of keratinocyte autophagy to increase inflammation may have important implications for the development of novel therapeutic strategies for improving inflammatory homeostasis in psoriasis patients.

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Disclosures

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